Effect of temperature reduction on myotonia in rat skeletal muscles in vitro

Lorna FANNING and Mary MacDERMOTT
Department of Physiology, Royal College of Surgeons in Ireland, 123 St. Stephen's Green, Dublin 2, Republic of Ireland

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INTRODUCTION

Myotonia, a symptom occurring in the myotonic syndromes, manifests itself as a failure of skeletal muscle to relax normally following voluntary contraction. This leads to slowing of relaxation, also described as an after-contraction, and is experienced by the patient as muscle stiffness. Electromyographic (EMG) recordings from myotonic muscles exhibit myotonic runs, which are high frequency discharges occurring after the trains of voluntarily evoked action potentials. This uncontrolled electrical activity (myotonic runs) underlies the failure of the muscle to relax [1]. Myotonia occurs in myotonic dystrophy and also in the non-dystrophic syndromes of hyperkalaemic periodic paralysis, paramyotonia congenita and myotonia congenita. The symptom of myotonia manifests itself in its purest form in myotonia congenita. The underlying defect in autosomal recessive generalized myotonia congenita in humans [2], and in the similar myotonia in goats [3], is a decrease in the chloride conductance, GCl, of the muscle fibre membrane. As a result of the decrease in GCl, repetitive electrical activity occurs in response to a brief stimulus or following voluntary contraction, leading to failure of the muscle to relax. There is confusion in the literature about the precise effect of muscle cooling on myotonic contractions in myotonia congenita. In humans suffering from recessive generalized myotonia congenita, Ricker et al. [4] recorded tetanic contractions in the adductor pollicis muscle when the muscle temperature was 33°C and after it had been maintained at 20°C for 30 min by fixing the hand and arm in a vessel of iced water. At a temperature of 33°C, measured using an EMG thermo needle, the recordings showed an after contraction typical of myotonia, whereas at 20°C the after-contraction no longer appeared, indicating that myotonia was alleviated at low temperature. In contrast, Walton and Gardner-Medwin [5]...
have stated, based on patient reports, that myotonia was aggravated at low temperatures. Likewise, in myotonic goats, the myotonia was observed to worsen in cold weather, while application of heat to the thighs of the goats appeared to improve the myotonia [6]. It is possible that the conflicting results are related to the fact that quantitative measurements were only made in the study of Ricker et al. [4]. In the other two studies [5, 6], the results were based on observation or subjective sensation, either or both of which could be unreliable.

The objective of the present study was to examine the effect of temperature reduction on chemically induced myotonia in rat muscles. A reproducible model of myotonia congenita was induced in rat muscles in vitro, using the chloride channel blocker anthracene-9-carboxylic acid (9-AC). 9-AC induces, both in vivo and in vitro, a model myotonia in which the changes in $G_{Cl}$ in the EMG tracing and in the relaxation time of the isometric twitch contraction, are similar to those which occur in the muscles of patients suffering from generalized recessive myotonia congenita [1]. A reliable quantitative measurement was used to assess the myotonic response in the muscles. The effect of temperature reduction on myotonia was determined by assessing the myotonic response to 9-AC at 37°C, 30°C, 25°C and 15°C.

At temperatures below 37°C, a decrease in membrane potential would be expected to occur. The effects of temperature reduction on myotonia could therefore be mediated by this decrease. To examine this possibility, the effect of changing the membrane potential of the muscles, by means other than temperature reduction, on their myotonic response was also determined. Membrane potentials and intracellular ionic activities were measured using microelectrodes.

The results of this study should help to clarify the situation regarding the effect of cold on myotonia of humans suffering from recessive generalized myotonia congenita, and therefore render the cold-exposure test more reliable. This test, together with neurological examination, EMG and other tests, can be used to classify myotonic patients. The results may also give valuable information about factors which influence membrane excitability and so further our knowledge of normal muscle function.

A brief report of some of the results of this work has been published [7].

METHODS

Male Wistar rats (250–300 g) were killed by cervical dislocation and the extensor digitorum longus (EDL) muscle was removed and immediately placed in a constant-temperature muscle-bath containing Krebs solution. The Krebs solution had the following composition (mmol/l): 120 NaCl, 25 NaHCO$_3$, 1.2 NaH$_2$PO$_4$, 1.2 MgSO$_4$, 5.0 KCl, 2.5 calcium gluconate, 11.5 glucose. It was bubbled continuously with 5% CO$_2$/95% O$_2$, and the pH was maintained at 7.4. Chloride-free Krebs solution was prepared by replacing all of the NaCl and KCl with equimolar amounts of sodium and potassium methyl sulphate. The K$^+$ concentration of the Krebs solution was increased by the addition of potassium chloride to normal Krebs or by the addition of potassium methyl sulphate to chloride-free Krebs. To record twitch contractions, the muscles were mounted vertically in the bath. One tendon was fixed to the base of the bath, while the other was attached to a strain gauge. The temperature was maintained constant using a Grant flow heater (Grant Instruments, Cambridge Ltd.) and recordings were made at either 37°, 30°, 25° or 15°C, so that each muscle was subjected to one temperature only. At each temperature the muscles were allowed to equilibrate in Krebs solution for 30 min before recordings were made. Each muscle was adjusted to its resting length and the stimulus strength and pulse width required to produce maximum twitch tension was then determined. Isometric twitch contractions were then elicited by applying supramaximal stimuli, of 0.5 ms duration, directly to the muscle. In each muscle, 4–5 twitches were elicited at 10 min intervals when the muscle was incubated in Krebs solution. Following the induction of myotonia by either the addition of $3 \times 10^{-5}$ mol/l 9-AC (Aldrich Chemical Co. Ltd.) to the Krebs solution, or following replacement of all of the Cl$^-$ of the Krebs solution with methyl sulphate, a further 4–5 twitches were elicited at 10 min intervals. For each muscle, the half-relaxation times, $T_{1/2}$, of the isometric twitches were measured, and mean values for $T_{1/2}$ before and after the addition of 9-AC or before and after replacement of the Krebs solution by Cl$^-$-free Krebs were calculated. The presence of myotonia was confirmed when $T_{1/2}$ after exposure to either of the myotonia-inducing agents was significantly greater than $T_{1/2}$ in Krebs solution. That the myotonic response can be quantified in terms of either the electrical or the mechanical activity was demonstrated in intact fibres exposed to 9-AC, and also in resected fibre segments from patients with myotonia congenita, where it was shown that the electrical and mechanical activities were highly correlated [1].

Intracellular potentials were measured in vitro when the muscles were incubated in Krebs solution. Resting membrane potentials, $V_m$, were measured using conventional 0.3 mol/l KCl-filled glass micro-capillary electrodes. The intracellular potassium activity was measured using K$^+$-sensitive micro-electrodes. The method for the fabrication of these micro-electrodes, their characteristics and the recording equipment has been described previously [8, 9].

The results of the contractile measurements are presented as means±SEM and the results of the
electrical potential measurements are presented as means ± 1 SD. Statistical significance was determined using Student's t-test, \( P < 0.05 \) being taken as significant.

RESULTS

Figure 1 (top) shows the variation with temperature of the mean (±SEM) \( T_{1/2} \) of isolated EDL muscles before and after the addition of \( 3 \times 10^{-5} \) mol/l 9-AC to the Krebs solution, and after replacement of Krebs solution by Cl⁻-free Krebs. \( T_{1/2} \) values measured in Krebs solution before replacement with Cl⁻-free Krebs solution were similar to those measured in Krebs before the addition of 9-AC. Consequently, for clarity, only the latter values are shown. It may be seen that before exposure to the myotonia-inducing agents, \( T_{1/2} \) in Krebs solution increased significantly as the temperature decreased. After the addition of \( 3 \times 10^{-5} \) mol/l 9-AC to the Krebs solution, the \( T_{1/2} \) values at 37°C, 30°C and 25°C were significantly higher than the \( T_{1/2} \) values before exposure to 9-AC, i.e. myotonia could be induced at each of these temperatures. At 15°C, however, myotonia could not be induced, in that 3 \( \times 10^{-5} \) mol/l 9-AC did not produce a significant increase in \( T_{1/2} \). A similar trend is evident when Krebs was replaced by Cl⁻-free Krebs solution. Myotonia could be induced at 37°C, 30°C and 25°C, but not at 15°C. The above results, expressed as the fold increase in \( T_{1/2} \) due to exposure to 9-AC or Cl⁻-free Krebs as a function of temperature, are shown in Fig. 1 (bottom). In this form, the results describe the degree of myotonia as a function of temperature. It is evident that at those temperatures at which myotonia could be induced, i.e. at 37°C, 30°C and 25°C, the degree of myotonia decreased as temperature decreased. However, examination of the relationships shown in Fig. 1 (top) indicate that this decrease is due to the increase which occurred in \( T_{1/2} \) in Krebs solution before exposure to the myotonia-inducing agents as the temperature decreased over this temperature range, rather than to any decrease in the responsiveness of the muscles to the myotonia-inducing agents. Elevation of the K⁺ concentration of the Krebs solution bathing the muscles to 7.5 mmol/l, or exposure of the muscles to 1 \( \times 10^{-4} \) mol/l ouabain, had no effect at any of the temperatures on \( T_{1/2} \), but after induction of myotonia both had marked effects. The effects of 7.5 mmol/l K⁺ and 1 \( \times 10^{-4} \) mol/l ouabain on the degree of myotonia induced by 3 \( \times 10^{-5} \) mol/l 9-AC as a function of temperature, are shown in Fig. 2. Raising the K⁺ concentration of the Krebs solution from 5 mmol/l to 7.5 mmol/l significantly reduced the degree of myotonia which developed at 37°C and reduced the myotonia to zero at lower temperatures. Ouabain at a concentration of 1 \( \times 10^{-4} \) mol/l abolished myotonia at the two temperatures at which its effect was examined, namely at 37°C and 30°C.

The membrane potential, \( V_m \), and the intracellular potassium activity, \( a_K \), were measured in EDL muscles in Krebs solution, before and after the addition of \( 3 \times 10^{-5} \) mol/l 9-AC, under the conditions shown in Table 1. The presence or absence of 9-AC had no effect on \( V_m \) or \( a_K \) under any of the conditions studied. Therefore, only the values for \( V_m \) or \( a_K \) obtained in the presence of 9-AC are shown in Table 1. In normal Krebs solution, the value of \( V_m \) measured at 25°C just failed to reach significance when compared with the value measured at 37°C. At 15°C, \( V_m \) was significantly lower than that measured at 37°C. This was probably due to loss of potassium and gain of sodium, but \( a_K \) measurements at 15°C were unsuccessful due to a
large increase in the resistance of the K⁺ electrodes at this temperature. Exposure to 1 × 10⁻⁴ mol/l ouabain at 37°C resulted in a significant membrane depolarization, accompanied by a significant decrease in aK⁺. Elevation of the K⁺ concentration of the Krebs solution to 7.5 mmol/l at 37°C also produced a significant depolarization.

The aK⁺ in the muscles exposed to this solution was higher than that in normal Krebs, but the increase was not significant.

**DISCUSSION**

This study has shown that the degree of myotonia induced by the Cl⁻–channel blocker 9-AC at 37°C was less pronounced at 30°C and at 25°C, and that myotonia failed to develop at 15°C (Fig. 1, bottom). The reason why the degree of myotonia which developed at 37°C became less pronounced at 30°C and 25°C (Fig. 1, bottom) was not due to any decrease in the responsiveness of the muscles to the myotonia-inducing agents as temperature decreased (Fig. 1, top). It was due to the increase in the Tr12, which occurred in the muscles before induction of myotonia as temperature decreased. An increase in Tr12 in rat skeletal muscles as the temperature decreases is well documented [10, 11]. At 15°C the myotonic response did not develop because the myotonia-inducing agents produced no increase in Tr12 (Fig. 1, top). The failure of 9-AC-induced myotonia to develop at 15°C could be explained if the ability of 9-AC to block Cl⁻–channels was reduced at 15°C, or if, at 15°C, other Cl⁻–channels opened which were not blocked by 9-AC. In either situation, the chloride conductance, GCl, would increase, which could prevent the development of the myotonic response. However, the results obtained in Cl⁻–free Krebs solution, where GCl could not have increased, suggest that this is an unlikely explanation. An increase in the potassium conductance, GK, at 15°C, by offsetting the effects of low GCl, could also prevent the development of myotonia. This too is unlikely, since GK in mammalian muscles shows little variation with temperature [12]. The delay in relaxation induced by 9-AC is due to repetitive firing of action potentials [1]. These arise from the negative after-potential which is slowed and prolonged in duration when GCl is reduced [13]. It seems fair to assume, therefore, that the non-appearance of myotonia at 15°C is due to cessation of the repetitive activity. This could be associated with the membrane depolarization which occurred in the muscles when the temperature was reduced to 15°C (Table 1).

A reduction in Vm of rat EDL muscles has been shown to result in a decrease in the amplitude and the conduction velocity of the action potential [14], which could affect the ability of the fibres to fire repetitively. Furthermore, it has been reported by Simoncini and Stuhmer [15] that depolarization of rat EDL muscles was accompanied by inactivation of voltage-dependant Na⁺ channels, and it was suggested by them that this could be responsible for the eventual termination of the myotonic discharge in myotonic patients. A role for membrane depolarization in preventing/reducing the myotonic response is

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**Table 1.** Vm and aK⁺ in myotonic EDL muscles under various conditions and measured using microelectrodes. Myotonia was induced by exposure to 3 × 10⁻³ mol/l 9-AC. Values are means ± SD; 4-6 muscles were examined under each set of conditions. The total number of fibres impaled is given in parentheses. *Membrane potential significantly lower than that of control. †Intracellular potassium activity significantly lower than that of control.

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<td>Vm (mV)</td>
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<td>aK⁺ (mmol/l)</td>
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Temperature reduction and myotonia

supported by the observations that the other two conditions studied under which myotonia was reduced significantly, i.e. elevation of extracellular K+ at 37°C and exposure to ouabain at 37°C (Fig. 2), were also associated with membrane depolarization. It could be argued that depolarization under these conditions will be associated with a change in the intracellular K+ activity, \( a_K \), and that this, rather than membrane depolarization, could be important in preventing/reducing the development of myotonia. At 15°C, due to Na+/K+ pump inhibition, a low \( a_K \) would be expected, but this could not be confirmed for the reason mentioned in the Results section. However, in the muscles exposed to 7.5 mmol/l K+, \( a_K \) was unchanged (Table 1), suggesting that changes in \( a_K \) are not an important factor in preventing/reducing the myotonic response. The effect of elevating the K+ concentration on the myotonic response which was observed in this study is in agreement with that previously reported [16], where it was noted that myotonia induced in rat diaphragm by 2,4-dichlorophenoxyacetate, was also reduced by 7.5 mmol/l K+. Although the membrane had probably depolarized, \( V_m \) was not measured. Support for the suggestion that membrane depolarization may prevent or reduce the myotonic response can be found in the literature. The diaphragm of alloxan-treated diabetic mice failed to develop myotonia in response to 9-AC. The diabetic fibres were shown to be depolarized, but following repolarization with insulin, sensitivity to 9-AC returned [17]. Similarly, myotonia induced by 2,4-dichlorophenoxyacetate in rat diaphragm failed to develop in muscles previously denervated [16], and depolarization of muscle after denervation is well documented [18-20]. Finally, other studies in this laboratory have shown that failure of rat muscles to develop myotonia \textit{in vivo}, following a period of water deprivation, was also associated with membrane depolarization [21].

The present study, as well as others, has demonstrated a link between depolarization and failure of myotonia to develop. In view of the results of Simoncini and Stuhmer [15], it is likely that depolarization prevents the development of myotonic contractions by inactivating the voltage-dependent Na+ channels. The failure of 9-AC-induced myotonia to develop at low temperatures supports the results of Rickers et al. [4] obtained in human myotonic patients, which showed that myotonic contractions of the adductor pollicis muscle reverted to normal when the muscle temperature was lowered. Myotonia was alleviated in humans at 20°C, but in the present study it was not possible to identify the precise temperature at which myotonia failed to develop. Contractile measurements in the presence of 9-AC were made only at 37°C, 30°C, 25°C and 15°C and at none of the intermediate temperatures. Consequently, since myotonia developed at all temperatures except 15°C, the results indicate that 9-AC-induced myotonia failed to develop at some temperature between 15°C and 25°C. There is evidence to suggest that this temperature may not be very much lower than 25°C. This follows from the other results of this study which suggest a strong association between membrane depolarization and failure of myotonia to develop. At 25°C, \( V_m \) in the myotonic muscles was considerably lower than that measured at 37°C, although the decrease just failed to reach significance. It seems fair to suggest therefore that a small decrease in temperature below 25°C would probably have resulted in significant depolarization and presumably in failure of myotonia to develop. So it is probable that the temperature at which 9-AC-induced myotonia did not develop was in reasonable agreement with the temperature of 20°C at which human myotonic contractions disappeared.

Our results do not agree with the reported worsening of the myotonic signs at low temperatures in humans suffering from myotonia [5] and in myotonic goats [6]. It is difficult to assess these reports as they were not based on the results of any measurements but rather on observations. Furthermore, the low temperatures referred to were not quantified. It may be for these reasons that they are in conflict with the results of the only objective study done in humans [4] and our own results reported here, both of which are the outcome of quantitative measurements.

In conclusion, this study has shown that the conditions under which 9-AC-induced myotonia either did not develop or was significantly reduced, i.e. at a temperature of 15°C, after elevation of the K+ concentration of the Krebs solution to 7.5 mmol/l or exposure to \( 1 \times 10^{-4} \) mol/l ouabain, were each associated with membrane depolarization. It is suggested that depolarization may prevent the repetitive firing typical of myotonia by inactivation of voltage-dependant Na+ channels, and so prevent the myotonic response. The results of the study support those obtained in patients suffering from myotonia congenita, where the myotonic contractions reverted to normal when the muscle was cooled to 20°C.

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